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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

003300-798

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

**09/889242**

INTERNATIONAL APPLICATION NO.  
PCT/SE00/00073

INTERNATIONAL FILING DATE  
14 January 2000

PRIORITY DATE CLAIMED  
15 January 1999

TITLE OF INVENTION

**A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND USE OF SAID METHOD**

APPLICANT(S) FOR DO/EO/US

**PETER ERIKSSON and OWE ORWAR**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
- ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
- ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
- ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Transmittal of Formal Drawing

A certified copy of Swedish Application No. 9900134-9, filed 15 January 1999 was submitted during the international phase of examination. Thus the claim for priority has been perfected.

U.S. APPLICATION NO. (if known) see 37 CFR 1.50) <b>09/889242</b>	INTERNATIONAL APPLICATION NO. PCT/SE00/00073	ATTORNEY'S DOCKET NUMBER 003300-798
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17. ☒ The following fees are submitted:

CALCULATIONS

PTO USE ONLY

**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO ..... \$1,000.00 (960)

International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00 (970)

International preliminary examination fee (37 CFR 1.482) not paid to USPTO  
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00 (958)

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00 (956)

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00 (962)

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$ 1,000.00

Surcharge of \$130.00 (154) for furnishing the oath or declaration later than  
months from the earliest claimed priority date (37 CFR 1.492(e)).

20 ☐ 30 ☐

\$

Claims	Number Filed	Number Extra	Rate
Total Claims	45 -20 =	25	X\$18.00 (966)
Independent Claims	2 -3 =	0	X\$80.00 (964)
Multiple dependent claim(s) (if applicable)			+ \$270.00 (968)

\$ 450.00

\$ --

\$ --

**TOTAL OF ABOVE CALCULATIONS =**

\$ 1,450.00

Reduction for 1/2 for filing by small entity, if applicable (see below).

\$ 725.00

**SUBTOTAL =**

\$ 725.00

Processing fee of \$130.00 (156) for furnishing the English translation later than  
months from the earliest claimed priority date (37 CFR 1.492(f)).

20 ☐ 30 ☐

\$

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+

**TOTAL NATIONAL FEE =**

\$ 725.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by  
an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 (581) per property

\$ 40.00

**TOTAL FEES ENCLOSED =**

\$ 765.00

Amount to be:  
refunded \$

charged \$

- a. ☒ Small entity status is hereby claimed.
- b. ☒ A check in the amount of \$ 765.00 to cover the above fees is enclosed.
- c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of \$\_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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Filed: July 13, 2001

SIGNATURE

Benton S. Duffett, Jr.

NAME

22,030

REGISTRATION NUMBER

09/889242

JC18 Rec'd PCT/PTO 13 JUL 2001

Patent

Attorney's Docket No. 003300-798

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of ) BOX PCT  
PETER ERIKSSON et al ) Attention: DO/EO/US  
Application No. (Unassigned) ) Group Art Unit: Unassigned  
Filed: July 13, 2001 ) Examiner: Unassigned  
For: A METHOD FOR INTRODUCING )  
SUBSTANCES INTO CELLS, AND )  
USE OF SAID METHOD )

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

This is a national phase filing of International Application No. PCT/SE00/00073,  
filed January 14, 2000.

Prior to examination of the above-captioned patent application, kindly enter the  
following amendment.

**IN THE ABSTRACT:**

Please add the Abstract of the Disclosure that is provided on a separate sheet.

**IN THE CLAIMS:**

Kindly replace claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45, as follows:

1. (Amended) A method for introducing a substance comprising a nucleic acid  
into a mammalian neural stem cell or progenitor cell, wherein said nucleic acid directly

interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

4. (Amended) A method according to claim 3, wherein said substance comprises a single or double stranded, linear or circular DNA.

5. (Amended) A method according to claim 1, wherein said substance comprises a single or double stranded RNA.

6. (Amended) A method according to claim 1, wherein said substance is a fusion molecule comprising a nucleic acid part and a protein part.

7. (Amended) A method according to any claim 1, wherein said substance is an expression vector containing specific cDNA.

9. (Amended) A method according to claim 1, wherein said substance gives rise to a detectable signal.

13. (Amended) A method according to claim 11, wherein said detectable signal is due to a radioactively tagged nucleic acid.

14. (Amended) A method according to claim 1, wherein said cell is a cell in a tissue or cell culture.

15. (Amended) A method for identification of progenitor cells and/or stem cells comprising using the method according to claim 1.

16. (Amended) The method according to claim 15, wherein said cells after identification are isolated from surrounding cells of other types.

17. (Amended) A method for gene therapy comprising using the method according to claim 1.

18. (Amended) The method according to claim 6, wherein said protein part comprises a pharmaceutically active protein.

19. (Amended) A method for propagation of neural cells comprising using the method according to claim 8.

20. (Amended) The method according to claim 18, wherein said propagated neural cells are suitable for transplantation to patients.

21. (Amended) A method for detection of a medicinal product comprising cDNA containing expression plasmids comprising using the method according to claim 1.

22. (Amended) A method for diagnostic purposes comprising using the method according to claim 1.

23. (Amended) The method according to claim 8, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

24. (Amended) A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, wherein said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vivo, whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

26. (Amended) A method according to claim 24, wherein said substance comprises a single or double stranded, linear or circular DNA.

27. (Amended) A method according to claim 24, wherein said substance comprises a single or double stranded RNA.

36. (Amended) A method according to claim 1, wherein said cell is a cell in the central nervous system of a patient.

37. (Amended) A method for identification of progenitor cells and/or stem cells comprising using the method according to claim 24.

38. (Amended) The method according to claim 37, wherein said cells after identification are isolated from surrounding cells of other types.

39. (Amended) A method for gene therapy comprising using the method according to claim 24.

40. (Amended) A method according to claim 28, wherein said protein part comprises a pharmaceutically active protein.

41. (Amended) A method for propagation of neural cells comprising using the method according to claim 30.

42. (Amended) A method for detection of a medicinal product comprising cDNA containing expression plasmids comprising using the method according to claim 24.

43. (Amended) A method for diagnostic purposes comprising using the method according to claim 24.

44. (Amended) A method claim 35, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

45. (Amended) A method for treatment of neurological insult, disease, deficit or condition comprising using the method according to claim 24.

**REMARKS**

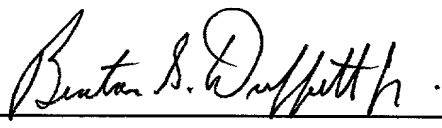
Entry of the foregoing amendments is respectfully requested.

The claim format is modified and multiple dependency is eliminated.

The examination and allowance of the application are respectfully requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By:   
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Date: July 13, 2001

### **Abstract of the Disclosure**

A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell, is disclosed. Also different applications of said method are disclosed.

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**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

1. (Amended) A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, [characterized in that] wherein said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

4. (Amended) A method according to [any one of the claims 1-3] claim 3, wherein said substance [is or] comprises a single or double stranded, linear or circular DNA.

5. (Amended) A method according to [any one of the claims 1-3] claim 1, wherein said substance [is or] comprises a single or double stranded RNA.

6. (Amended) A method according to [any one of the claims 1-3] claim 1, wherein said substance is a fusion molecule comprising a nucleic acid part and a protein part.

7. (Amended) A method according to any [one of the claims 1-3] claim 1, wherein said substance is an expression vector containing specific cDNA.

**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

9. (Amended) A method according to [any one of the claims 1-6] claim 1,  
wherein said substance gives rise to a detectable signal.
13. (Amended) A method according to claim 11 [or 12], wherein said detectable  
signal is due to a radioactively tagged nucleic acid.
14. (Amended) A method according to [any one of the claims 1-13] claim 1,  
wherein said cell is a cell in a tissue or cell culture.
15. (Amended) [Use of a] A method [according to any one of the claims 1-14,]  
for identification of progenitor cells and/or stem cells comprising using the method  
according to claim 1.
16. (Amended) [Use] The method according to claim 15, wherein said cells  
after identification [is] are isolated from surrounding cells of other types.
17. (Amended) [Use of a] A method [according to any one of the claims 1-14,]  
for gene therapy comprising using the method according to claim 1.

**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

18. (Amended) [Use of a] The method according to claim 6 [and 17], wherein said protein part [consists of] comprises a pharmaceutically active protein.

19. (Amended) [Use of a] A method [according to claim 8,] for propagation of neural cells comprising using the method according to claim 8.

20. (Amended) [Use] The method according to claim 18, wherein said propagated neural cells are suitable for transplantation to patients.

21. (Amended) [Use of a] A method [according to any one of the claims 1-14,] for detection of a medicinal product comprising cDNA containing expression plasmids comprising using the method according to claim 1.

22. (Amended) [Use of a] A method [according to any one of the claims 1-14,] for diagnostic purposes comprising using the method according to claim 1.

23. (Amended) [Use of a] The method according to [any one of the claims 8-13] claim 8, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

24. (Amended) A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, [characterized in that] wherein said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vivo, whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

26. (Amended) A method according to claim 24, wherein said substance [is or] comprises a single or double stranded, linear or circular DNA.

27. (Amended) A method according to claim 24, wherein said substance [is or] comprises a single or double stranded RNA.

36. (Amended) A method according to [any one of the claims 1-13] claim 1, wherein said cell is a cell in the central nervous system of a patient.

37. (Amended) [Use of a] A method [according to claim 24,] for identification of progenitor cells and/or stem cells comprising using the method according to claim 24.

38. (Amended) [Use] The method according to claim 37, wherein said cells after identification [is] are isolated from surrounding cells of other types.

**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

39. (Amended) [Use of a] A method [according to any one of the claims 24-38]  
for gene therapy comprising using the method according to claim 24.

40. (Amended) [Use of a] A method according to claim 28, wherein said  
protein part [consists of] comprises a pharmaceutically active protein.

41. (Amended) [Use of a] A method [according to claim 30,] for propagation of  
neural cells comprising using the method according to claim 30.

42. (Amended) [Use of a] A method [according to any one of the claims 24-36,]  
for detection of a medicinal product comprising cDNA containing expression plasmids  
comprising using the method according to claim 24.

43. (Amended) [Use of a] A method [according to any one of the claims 24-36,]  
for diagnostic purposes comprising using the method according to claim 24.

44. (Amended) [Use of a] A method [according to any one of the claims 30-35]  
claim 35, wherein said protein or detectable signal allows for testing or screening of  
aforementioned protein or signal.

**Attachment to Preliminary Amendment dated July 13, 2001**

**Marked-up Claims 1, 4 to 7, 9, 13 to 24, 26, 27 and 36 to 45**

45. (Amended) [Use of a] A method [according to claim 24,] for treatment of neurological insult, disease, deficit or condition comprising using the method according to claim 24.

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11/PR 75

A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND  
USE OF SAID METHOD

Field of the invention

The present invention relates to in vitro and in vivo methods for introducing substances into a mammalian stem cell and/or progenitor cells, as well as to use of such methods.

Background of the invention

For several years it has been clear that cellular mechanisms exist that allow cells to internalize nucleic acids. A new approach for chemotherapy has been developed based on the fact that addition of defined oligonucleotides (antisense inhibitors) to cells in tissue culture has been shown to block specific gene expression.

Previous studies have established that short single stranded DNAs are rapidly internalized by a variety of cultured cells (Bennett, R. M., Gabor, G. T. and Merritt, M. M., J. Clin. Invest. 76, 2182-2190 (1985); Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S. and Neckers, L. M., Proc. Natl. Acad. Sci. USA 86, 3474-3478 (1989); Yakubov, L. A., Deeva, E. A., Zarytova, V. F., Ivanova, E. M., Ryte, A. S., Yurchenko, L. V., and Vlassov, V. V., Proc. Natl. Acad. Sci. USA 86, 6454-6458 (1989); Iversen, P. L., Zhu, S., Meyer, A., and Zon, G., Antisense Res. Dev. 2, 211-222 (1992); Wu-Pong, S., Weiss, T. L., and Hunt, C. A. Pharmacol. Res. 9, 1010-1017 (1992); Chan, T. M., Framton, G and Cameron, J. S., Clin. Exp. Immunol. 91, 110-114 (1993)). There are reports of DNA receptor structures that mediate uptake and destruction of DNA in human leucocytes ((Bennett, R. M., Gabor, G. T. and Merritt, M. M. J. Clin. Invest. 76, 2182-2190 (1985))).

However, naked DNA, RNA and oligonucleotides are in general unable to cross cellular membranes in vivo (Bo-

ado, R. J., Tsukamoto, H. and Pardridge, W. M. J. Pharm. Sci. 87, 1308-1315 (1998). Therefore, several strategies based on the binding of DNA to soluble carriers, receptor structures or conjugates (e.g. DNA binding proteins, conjugates of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system (see e.g. WO 96/15811; WO 94/25608; Kato, Y. and Sugiyama, Y. Crit. Rev. Ther. Drug Carrier syst. 14, 287-331 (1997)).

In general, replacement of neurons following degeneration or damage is not a characteristic of the mammalian brain. Neuronal loss is thus considered permanent. Prolonged postnatal neurogenesis has been described in the granule cell layer of the hippocampal formation (Altman, J. and Das, G. D., J. Comp. Neurol. 124: 319-335 (1965); Altman, J. and Das, G. D., Nature 214: 1098-1101 (1967); Caviness, V. S. jr., J. Comp Neurol. 151: 113-120 (1973); Gueneau, G., Privat, A., Drouet, J., and Court, L., Dev. Neurosci. 5, 345-358 (1982); Eckenhoff, M. F. and Rakic, P., J. Neurosci. 8: 2729-2747 (1988)). Cell genesis and neurogenesis have recently been shown to persist well into adulthood in man (Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D. A., Gage, F. H., Nature Med. 4:1313-1317 (1998)).

Newborn neurons in the granule cell layer express markers of differentiated neurons and have morphological characteristics corresponding to differentiated granulae cells (Kaplan, M. S. and Bell, D. H., J. Neurosci. 4: 1429-1441 (1984); Cameron, H. A., Woolley, C. S., McEwen, B. S., and Gould, E., Neuroscience 56: 337-344 (1993); Cameron, H. A., Woolley, C. S., and Gould, E., Brain Res. 611: 342-346 (1993)). Furthermore, they establish axonal processes into the mossy fiber pathway and form synaptic connections with their targets in hippocampus CA3 (Seki, T. and Arai, Y., J. Neurosci. 13: 2351-2358 (1993); Stanfield, B. B., and Trice, J. E., Exp. Brain Res. 72: 399-406 (1988)). The hippocampus is associated with spatial

learning and memory (McNamara, R. K., and Skelton, R. W., Brain Res. Rev. 18: 33-49 (1993)). The proliferation of progenitor cells can be influenced by the administration of N-methyl-D-aspartate (NMDA) receptor antagonists or by the removal of the adrenal glands (Cameron, H. A., and Gould, E., Neuroscience 61: 203-209 (1994); Cameron, H. A., Tanapat, P., and Gould, E., Neuroscience 82: 349-354 (1998)). Plasticity is reduced with increasing age, and recent studies have demonstrated that proliferation of progenitor cells also is decreased but not completely abolished with age (Kuhn, H., Dickinson-Anson, H., and Gage, F. H., J. Neurosci. 16: 2027-2033 (1996)). Stem cells, isolated through a time consuming and laborious tissue culture procedure, from the adult rodent brain has recently been transplanted into the brain of adult animals where they differentiate into cells with neuronal characteristics (Suhonen, J. O., Peterson, D. A., Ray, J., and Gage, F. H., Nature 383:624-627 (1996)). There are so far no known stem cell markers that are usable for rapid isolation of stem cells or progenitor cells from the adult central nervous system. This fact inhibits the therapeutic use of stem cells in humans. So far the detection of stem cells rely on indirect detection method using modified nucleotides that incorporates in to the genome in dividing cells during the S-phase of the cell cycle. Thereafter, the phenotype of the progeny can be detected using immunohistochemical methods. The limitation with this way of identifying progenitor progeny is that these cells no longer possess the stem cell or progenitor cell properties meaning that these cells lack the ability to self renew and to give rise to neurons, astroglia, or oligodendrocytes. Alternative strategies to isolate stemcells from rodents, based on either unselective dye staining, immunosorting with antibodies against the protein nestin expressed by all cells surrounding the ventricles, or unselective infection with viruses carrying the gene for a selectable marker, was recently pub-

lished (Johansson, C. B., Momma, S., Clarke, D. L., Ris-  
ling, M., Lendahl, U., Frisen, J., Cell 96: 25-34  
(1999)). Neither of these methods is highly efficient and  
thus unsuitable for rapid isolation of stemcells from  
5 small human tissue samples. Therefore, it is of impor-  
tance to identify a usable marker or property allowing  
for rapid isolation of stem or progenitor cells for  
therapeutic purposes e.g. autologous neural transplanta-  
tion.

10

#### Summary of the invention

Due to the fact that mammalian progenitor cells and  
stem cells from the adult CNS lack specific marker mole-  
cules it has up to now been virtually impossible to per-  
15 form rapid detection and isolation of those cells. During  
the work leading to the present invention it was found  
that progenitor cells and stem cells from the adult brain  
possess a highly efficient mechanism for uptake of nu-  
cleic acids, such as DNA. It was also found that it is  
20 possible to use said transport system in order to mark or  
tag progenitor cells and stem cells via administration of  
e.g. double stranded DNA either in linear form or in cir-  
cular form (plasmids) which is taken up by the progenitor  
cells or the stem cells via direct interaction between  
25 the DNA and the cells, without the use of facilitating  
drugs, carriers, soluble receptors or chemicals or any  
special devices. The DNA is not immediately degraded. In-  
stead, if the plasmid DNA contains the necessary compo-  
nents for expression, the aforementioned cells can be de-  
30 tected by the expression of plasmid cDNAs. If the  
DNA/plasmid, containing suitable elements for expression,  
cDNA and promotor, is incubated in the presence of pro-  
genitor cells or stem cells, said DNA is taken up effi-  
ciently and the protein corresponding to the cDNA is ex-  
35 pressed by the progenitor cells or stem cells.

The invention is based on the use of this nucleic  
acid transport system in progenitor cells and stem cells

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for different purposes. According to the present invention, it is possible to transfer DNA without the help or aid of e.g. viral vectors. The invention provides new methods to isolate progenitor cells and stem cells in vivo and in vitro. This isolation may be based on the expression from plasmid containing cDNA of a protein that enables selective identification and isolation based on immunoreactivity, or on the expression by DNA of a protein that enables selective identification and isolation based on the expression of fluorescent proteins, including FACS sorting. The invention also provides new methods to transport different substances with e.g. pharmaceutical effects into progenitor cells and/or stem cells.

The object of the invention is thus a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell. Said method may be performed both in vitro and in vivo.

The method is particularly suitable for isolation of progenitor cells or stem cells from the adult brain, for gene therapy, for cell sorting and for diagnostic procedures.

The characterizing features of the invention will be evident from the following description and the appended claims.

There are several advantages with the present invention compared with known strategies based on the binding of DNA to a soluble carrier, a receptor structure or a conjugate (such as a DNA binding protein, a conjugate of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system. One important advantage is that the invention does not rely on the binding of DNA to any soluble receptors or

carriers. Another important advantage is that it allows for the selective labeling of cells, due to the fact that only cells with the above described inherent transport mechanism are transfected.

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Detailed description of the invention

The transport mechanism upon which the present invention is based and which is found in mammalian stem cells and progenitor cells from the brain, including human stem cells and progenitor cells from the brain, can be utilized in order to transport single or double stranded DNA or RNA into a cell and subsequently allow for the DNA or RNA to remain intact and undegraded in the cell. Cells in which this transport mechanism is found are especially adult derived neural stem cells and progenitor cells.

By utilizing this transport it is thus possible to insert nucleic acids into said cells.

These nucleic acids may either be used for their ability to make it possible to identify and thus isolate progenitor cells and stem cells from other cells, or for their pharmaceutical effects.

As stated above, the present invention relates to a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said substance is brought into contact with said cell, whereby it is taken up by the cell via the inherent transport mechanism of the cell. The method may be used both in vitro and in vivo. The cells used in the method according to the invention are preferably derived from an adult.

The substance to be introduced into a cell according to the method is or comprises e.g. a single or double stranded, linear or circular DNA, or a single or double stranded RNA. The substance may also be a fusion molecule comprising a nucleic acid part and a protein part, or an expression vector containing a specific cDNA. The expres-

sion "expression vector" used herein relates to all vectors or plasmids consisting of a double stranded DNA structure comprising cDNA for a specific peptide or protein. Once this expression vector is taken up by the stem cells or progenitor cells it will lead to the synthesis of said peptide or protein.

When the substance is an expression vector it is preferably, according to one embodiment of the invention further commented on below, that the cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

As stated above, the method according to the invention may be performed both in vitro, e.g. in a tissue or cell culture, and in vivo. When the method is performed in vivo, the cells into which the substance is transported are preferably cells in the central nervous system.

The methods according to the invention may be used for several different purposes, both diagnostic and therapeutic.

When the method is performed in vitro, it is especially suitable for the identification of progenitor cells and stem cells. When the methods according to the invention are used for the purpose of identification it is preferable that the substance that is to be introduced into said cells gives rise to a detectable signal or to a peptide or protein that enables selective identification of stem cells and progenitor cells. Said peptide or protein may then in its turn give rise to a detectable signal, as the case is for e.g. a fluorescent protein, or a marker protein. Examples of suitable markers for stem cells or progenitor cells are protein components of the transport system, such as receptors and carriers. The detectable signal may also be obtained by the use of tagged substances, such as a radioactively tagged nucleic acid.

It is especially interesting to be able to identify, and thereafter isolate, progenitor cells and stem cells in samples constituted of e.g. different structures of brain tissue taken out of a patient or cells cultured from a patient.

Once the stem cells or progenitor cells have been identified, they can be isolated from the other types of cells in the sample by any appropriate method known to man skilled in the art. The isolated cells can then for example be used in different tests, for diagnostic purposes or be propagated and transplanted to a patient.

The in vivo method can be used in order to identify, and subsequently isolate, cells in vivo, in a way similar to the in vitro method described above. When the method is performed in vivo, it is possible to identify, and thus isolate, stem cells and progenitor cells in different structures of the intact brain

It is also possible to propagate stem cells and progenitor cells with the methods according to the invention. This propagation can be performed both in vitro and in vivo. The cells, which optionally first may have been identified and isolated with the methods according to the invention, are then brought in contact with a substance that comprises or gives rise to peptide or protein that, once it is taken up by the cells, activate proliferation and/or differentiation and/or lineage determination of said cells.

It is also possible to use both the in vitro and the in vivo methods according to the invention for gene therapy. The substance that is brought into contact with the cells, and subsequently is transported into the cells, may then be a pharmaceutically active substance. It may also give rise to a pharmaceutically active substance once it is taken up by the cells. The substance may then e.g. be an expression vector comprising cDNA encoding the pharmaceutically active substance. The pharmaceutically active substance produced by the cDNA once it is taken up

by the cell may be a peptide or protein that will get transported out of the progenitor cell or stem cell to affect surrounding tissue or cells. Examples of such a peptide or protein are trophic factors, or other proteins exerting a desired action on neighboring cells and tissues. The peptide or protein produced by the cDNA may also be a substance that will either activate or inactivate proliferation, differentiation or specific lineage determination of the progenitor cells or stem cells either in order to be able to more easily isolate progenitors or stem cells or in order to induce the genesis of new neurons, astrocytes or oligodendrocytes from progenitors or stem cells in the brain or within progenitors or stem cells in a tissue culture for concomitant use for transplantation of said cells to patients. It is also possible to use a substance constituted of a fusion molecule between a nucleic acid, that enables the transport into the cells, and a pharmaceutically active protein.

When the gene therapy is performed in vivo, it can be used for treatment of neurological insult, disease, deficit or condition in a patient. The term "treatment" used herein relates to both treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The treatment may either be performed in an acute or in a chronic way. The term "patient", as it is used herein, relates to any human or non-human mammal in need of treatment according to the invention.

It is possible to produce medicinal products for treatment of conditions due to disturbances of the normal function of stem cells or progenitor cells by attaching a pharmaceutically active compound to a nucleic acid. The nucleic acid will, when it is brought into contact with a stem cell or progenitor cell, be taken up into the cell by the inherent transport mechanism of the cell, and since the pharmaceutically active compound is attached to the nucleic acid it too will be transported in to the

cell. Such medicinal products may also comprise other substances, such as an inert vehicle, or pharmaceutical acceptable adjuvants, carriers, preservatives etc., which are well known to persons skilled in the art. It is preferable that such medicinal products are administered to a patient by infusion into the cerebral ventricles through a surgically inserted canula or via a syringe inserted between lumbar vertebrae and into the spinal fluid.

The methods according to the invention can also be used in order to test or screen a protein or a detectable signal. In a screening or test application the invention is used in with stemcells that take up DNA including cDNA coding for a protein of interest that are subject to screening or testing. Examples of proteins are receptors that can be used for screening new receptor agonists. The transport and uptake of and subsequent expression from plasmids in cells according to the invention can be used in detector devices and screening devices where expression of specific proteins like receptors or enzymes are desired. The advantage of the present invention compared with conventional transfection techniques in which drugs or compounds that facilitate DNA uptake are necessary for efficient uptake and expression, is the high efficiency and lack of need for drugs, compounds or chemicals to facilitate uptake and subsequent expression of proteins.

The invention will now be further explained in the following example. This example is only intended to illustrate the invention and should in no way be considered to limit the scope of the invention.

#### Brief description of the drawing

In the example below, reference will be made to the accompanying figure, wherein:

Figure 1A is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of a plasmid containing the cDNA for GFP;

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Figure 1B a lightmicroscopic image showing the same result as figure 1A;

Figure 1C is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of another plasmid not containing the GFP gene; and

Figure 1D a lightmicroscopic image showing the same result as figure 1B.

### Example

#### Expression by progenitor cells from mature rat brain of a fluorescent marker

The expression of green fluorescent protein (GFP) was examined in progenitor cells isolated from the adult hippocampus. Progenitor cells (Palmer, T. D., Ray, J. and Gage, F. H. (1995) Mol. Cell. Neurosci. 6: 474-486.) and cos-7 cells were cultured according to standard procedures and plated onto 1-inch circular coverslips coated with poly-D-ornithine and lamilin. The cells were incubated with plasmids containing the cDNA for GFP, and plasmids deficient of the GFP gene, respectively, in a humid atmosphere at 37°C with 5% CO<sub>2</sub> and 95% air for 10 minutes. The cells were cultured for 48 h, following DNA exposition.

Thereafter the expression of the fluorescent protein was detected using an inverted Leica DMIRB microscope equipped for fluorescence microscopy. The cells were viewed in the microscope using excitation of GFP at 488 nm using an Ar-ion laser (Spectra Physics model 2025-05, Sunnyvale, CA). The laser light was sent through a 488-line interference filter followed by a spinning disk to break the coherence and scatter the laser light. The laser was collected by a lens and sent through a fluorescein filter cube (Leica I-3) into the objective to excite the fluorophores. The resulting fluorescence was collected by the same objective and the image was detected by a 3-chip color CCD-camera (Panasonic) and recorded at

25 Hz frame collection rate by a Super VHS (Panasonic SVHS AG-5700). The CCD images were digitized from tape and processed for presentation.

When progenitor cells were incubated in medium with 50  $\mu\text{g}/\text{ml}$  of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, they were highly fluorescent. Figure 1A is a fluorescence photomicrograph showing this result, and Figure 1B shows the respective lightmicroscopic image.

In contrast, when progenitor cells from adult rat brain were incubated with other plasmids not containing the GFP gene, no fluorescence was observed. Figure 1C and 1D show the respective images when GFP deficient plasmid DNA were used.

Also, when progenitor cells were incubated with plasmid containing the gene expressing b-galactosidase 50  $\mu\text{g}/\text{ml}$ , without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, cells expressed b-galactosidase activity.

It was also found that kidney-derived Cos-7 cells that were incubated in medium with 50  $\mu\text{g}/\text{ml}$  of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, lack expression of green fluorescent protein (GFP). Detection and experimental procedures for this experiment was identical to that for progenitor cells exposed to plasmid containing the cDNA for GFP.

Also, when cos-7 cells were incubated with plasmid containing the gene expressing b-galactosidase, without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, they displayed a lack of expression of b-galactosidase activity.

It is clear from the above experiments that progenitor cells from adult rat brain has a capacity to in vitro transport double-stranded DNA plasmids to their interiors, and to synthesize the proteins that the DNA sequence

5 codes for.

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CLAIMS

1. A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

2. A method according to claim 1, wherein said cell is derived from an adult.

3. A method according to claim 2, wherein said method is performed in a humid atmosphere at 37°C.

4. A method according to any one of the claims 1-3, wherein said substance is or comprises a single or double stranded, linear or circular DNA.

5. A method according to any one of the claims 1-3, wherein said substance is or comprises a single or double stranded RNA.

6. A method according to any one of the claims 1-3, wherein said substance is a fusion molecule comprising a nucleic acid part and a protein part.

7. A method according to any one of the claims 1-3, wherein said substance is an expression vector containing a specific cDNA.

8. A method according to claim 7, wherein said cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

9. A method according to any one of the claims 1-6, wherein said substance gives rise to a detectable signal.

10. A method according to claim 7, wherein said cDNA gives rise to a peptide or protein that enables selective identification of stem cells and/or progenitor cells.

11. A method according to claim 10, wherein said peptide or protein gives rise to a detectable signal.

12. A method according to claim 11, wherein said protein is a fluorescent protein.

13. A method according to claim 11 or 12, wherein said detectable signal is due to a radioactively tagged nucleic acid.

14. A method according to any one of the claims 1-13, wherein said cell is a cell in a tissue or cell culture.

15. Use of a method according to any one of the claims 1-14, for identification of progenitor cells and/or stem cells.

16. Use according to claim 15, wherein said cells after identification is isolated from surrounding cells of other types.

17. Use of a method according to any one of the claims 1-14, for gene therapy.

18. Use of a method according to claim 6 and 17, wherein said protein part consists of a pharmaceutically active protein.

19. Use of a method according to claim 8, for propagation of neural cells.

20. Use according to claim 18, wherein said propagated neural cells are suitable for transplantation to patients.

21. Use of a method according to any one of the claims 1-14, for detection of a medicinal product comprising cDNA containing expression plasmids.

22. Use of a method according to any one of the claims 1-14, for diagnostic purposes.

23. Use of a method according to any one of the claims 8-13, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

24. A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or

a component within said cell membrane in vivo, whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

5           25. A method according to claim 24, wherein said cell is derived from an adult.

          26. A method according to claim 24, wherein said substance is or comprises a single or double stranded, linear or circular DNA.

10           27. A method according to claim 24, wherein said substance is or comprises a single or double stranded RNA.

          28. A method according to claim 24, wherein said substance is a fusion molecule comprising a nucleic acid  
15           part and a protein part.

          29. A method according to claim 24, wherein said substance is an expression vector containing a specific cDNA.

          30. A method according to claim 29, wherein said  
20           cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

          31. A method according to claim 24, wherein said substance gives rise to a detectable signal.

25           32. A method according to claim 29, wherein said cDNA gives rise to a peptide or protein that enables selective identification of stem cells and/or progenitor cells.

          33. A method according to claim 32, wherein said  
30           peptide or protein gives rise to a detectable signal.

          34. A method according to claim 33, wherein said protein is a fluorescent protein.

          35. A method according to claim 33, wherein said de-  
35           tectable signal is due to a radioactively tagged nucleic acid.

36. A method according to any one of the claims 1-13, wherein said cell is a cell in the central nervous system of a patient.

37. Use of a method according to claim 24, for identification of progenitor cells and/or stem cells.

38. Use according to claim 37, wherein said cells after identification is isolated from surrounding cells of other types.

39. Use of a method according to any one of the claims 24-38 for gene therapy.

40. Use of a method according to claim 28, wherein said protein part consists of a pharmaceutically active protein.

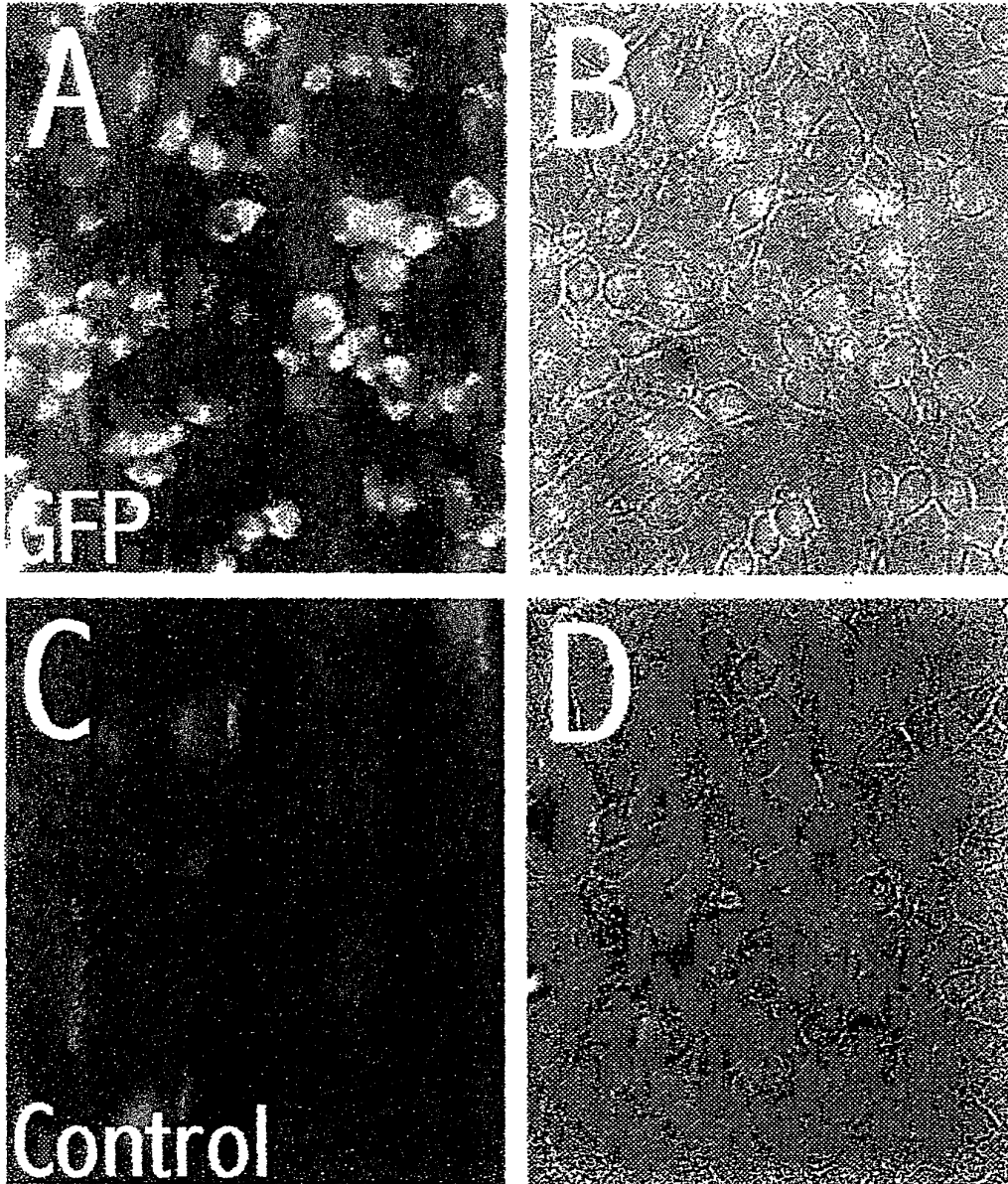
41. Use of a method according to claim 30, for propagation of neural cells.

42. Use of a method according to any one of the claims 24-36, for detection of a medicinal product comprising cDNA containing expression plasmids.

43. Use of a method according to any one of the claims 24-36, for diagnostic purposes.

44. Use of a method according to any one of the claims 30-35, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

45. Use of a method according to claim 24, for treatment of neurological insult, disease, deficit or condition.

**Fig. 1**

**COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR UTILITY PATENT APPLICATION**

Attorney's Docket No.

003300-798

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS. AND USE OF SAID METHOD

the specification of which

(check one)

☐ is attached hereto;

☒ was filed on 14 January 2000 as

Application No. PCT/SE00/00073

and was amended on \_\_\_\_\_;  
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
Sweden	9900134-9	15 January 1999	YES <u>x</u> NO <u>  </u>
			YES <u>  </u> NO <u>  </u>

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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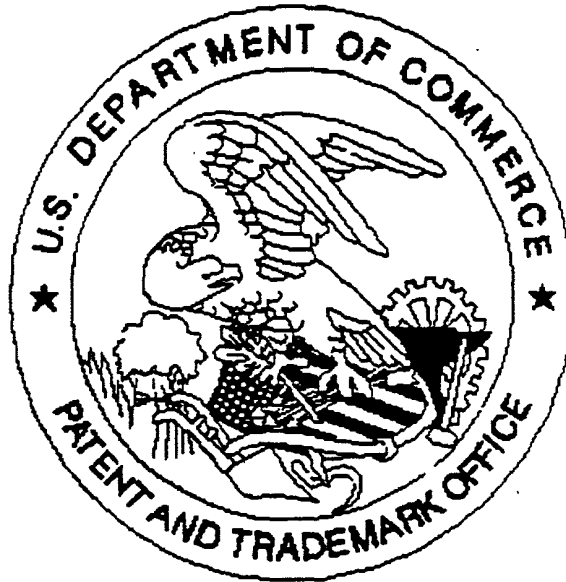
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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